Research article

Visual and simple determination of glucose-induced acidification by yeast cells: application to rapid cytotoxicity test

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A B S T R A C T

This study demonstrated that glucose-induced proton release from yeast cells was more sensitive to various inhibitors than cell proliferation. In this study the inhibition of glucose-induced proton release was determined on the basis of color change of pH indicator, methyl red, from pH 5 to pH6 at cell density of 2.5×10^7 cells/ml. When yeast cells were incubated with the inhibitors of glucose intake, glycolysis, and plasma membrane H^+-ATPase for 1 h, these cytotoxic effects were observed by following the change in absorbance at 527 nm due to methyl red for 5 min. The cytotoxic effects of heavy metal ions, detergents and quinones were observed in the same manner. The above method was superior in sensitivity and measurement time to cell proliferation measurement that required 9 h. This visual cytotoxicity test (methyl red test) is expected to be useful as simple and rapid cytotoxicity test with yeast cells.

1. Introduction

The mechanism and measuring method of glucose-induced acidification in yeast cell suspension have been studied in the field of biochemistry, microbiology and brewing. Glucose-induced acidification was found to depend on plasma membrane H^+-ATPase which plays an important role in proton efflux from yeast cells (Na et al., 1993; Viegas et al., 1995; Ambesi et al., 2000; Lecchi et al., 2005), while the dissociation of hydrated carbon dioxide and the release of organic acid were also reported to contribute to acidification in high density yeast cell suspension (Sigler and Hoffer, 1991; Myers et al., 2005). The measuring method of glucose-induced acidification with pH meter was improved to evaluate the yeast vitality in brewing practice (Sigler et al., 2006; Gabriel et al., 2008a), and the colorimetric methods also has been proposed (Pascual and Kotyk, 1982; Gabriel et al., 2008b).

The purpose of this study is to propose that the measuring method of glucose-induced acidification can be applied not only to the measurement of yeast activity in fermentation but also to the detection of toxic substances that damage yeast. This study suggests the improved colorimetric method to evaluate the inhibitory effects of the cytotoxic compounds on glucose-induced acidification. In this study methyl red was used as pH indicator in order to determine the acidification from pH 6 to pH 5 at low cell density within 5 min. This rapid colorimetric assay

(methyl red test) was demonstrated to be superior in the detection of the cytotoxic compounds to cell proliferation measurement requiring 9 h. Therefore, methyl red test is expected to be useful as the rapid cytotoxicity test.

2. Materials and methods

2.1. Yeast strain and cultivation

Baker yeast cells *Saccharomyces cerevisiae* IFO2044 were supplied from National Institute of Technology and Evaluation in Japan. The cells were grown in test tube filled with YPD medium (2% glucose, 1% peptone, and 0.5% yeast extract) at 30 °C for 15 h. The test tube was degassed and sealed during the cultivation and stirred occasionally. The cells were washed two times with distilled water by centrifugation, and the absorbance of yeast cell suspension was adjusted to 1.0 at 600 nm with distilled water. This absorbance corresponded to the cell density of 2.5×10^7 cell/ml, and the percentage of wet weight/volume was 0.88%. The yeast cell suspension was stored at room temperature before the determination of glucose-induced acidification.

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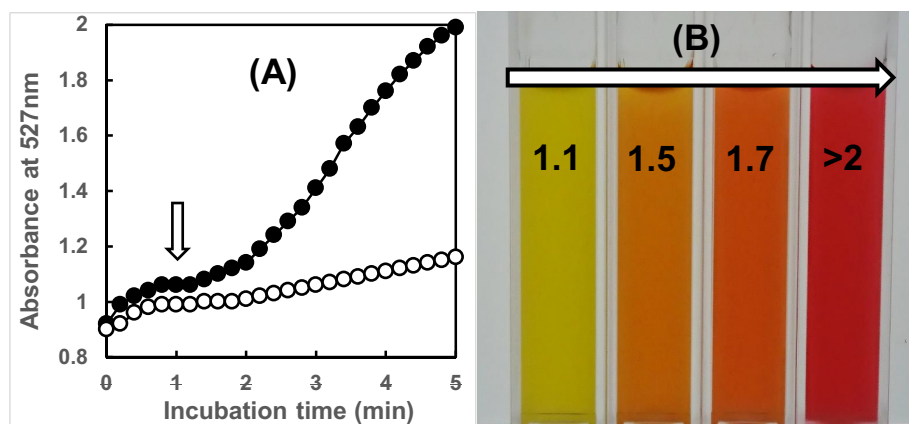
Figure 1. Change in absorbance of methyl red during glucose-induced acidification in yeast cell suspension. Arrow in (A) shows the addition of 20 mM glucose. Symbols ● and ○ shows no addition and addition of 1,4-naphthoquinone, respectively. Each symbol represents the mean of three different determinations, and the standard deviation was less than 6% of the mean. In (B) arrow shows the direction of glucose-induced acidification, and number in photo corresponds to the absorbance at 527 nm.

2.2. Determination of glucose-induced acidification by methyl red (methyl red test)

Yeast cell suspension was incubated for 1 min after 1.0 ml of yeast cell suspension was mixed with 8μl of 10 mM methyl red dissolved in dimethyl sulfoxide, 20μl of 1M KCl, and 1.2μl of 0.1N NaOH. After stirring of the above mixture, 20μl of 1M glucose was added to the yeast cell suspension, and the change in absorbance at 527 nm was recorded for 5 min. The change in pH was also measured by a pH-meter with microelectrode.

2.3. Cytotoxicity test

Test solution of 100 μl or less was mixed with 1.0 ml of yeast cell and was incubated at 30 °C for 1 h. The mixture was then centrifuged at 6000 rpm for 2 min, and the sediment was suspended with 1.0 ml of distilled water. Yeast cells were twice washed with 1.0 ml of distilled water, and the precipitated yeast cells were re-suspended with 1.0 ml of distilled water. The yeast cell suspension was used for the determination of glucose-induced acidification as described in 2.2. Intact yeast cells were also washed under the above conditions.

2.4. Cell proliferation measurement

After 1 ml of yeast cell suspension containing 2.5×10^6 cells in YPD medium was mixed with test solution of 100 μl or less, the increasing turbidity of yeast culture at 30℃# was determined by following the absorbance at 600 nm for 9 h.

2.5. Chemicals

All the chemicals were obtained from Fuji Film and Wako Pure Chemical Industry, Ltd.

The metal ions were dissolved in distilled water, and the organic compounds were dissolved in dimethyl sulfoxide.

2.6. Statistical analysis

Each experiment was repeated three times, and the mean values ± the standard deviation were calculated using Microsoft Office Excel software 2016 version and presented in the figures.

3. Results and discussion

3.1. Glucose-induced acidification

Though bromocresol green was used as pH indicator for acidification power test in the pH range from 3.5 to 5.3 (Gabriel et al., 2008b), methyl red was used in order to detect the lower proton release at pH range from 5 to 6 in this study.

Figure 1 (A) shows the change in absorbance at 527 nm before and after the addition of glucose to yeast cell suspension. The increase in absorbance was observed after the addition of KCl and stopped in 1 min
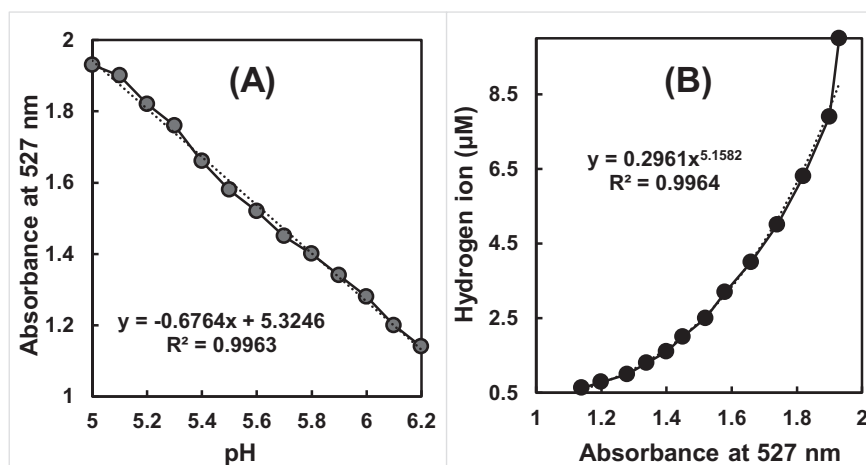

Figure 2. Correlation among absorbance, pH and the calculated concentration of H^+. (A) shows the correlation between absorbance and pH. (B) shows the correlation between absorbance and the concentration of H^+ calculated from pH = -log[H^+]. Each symbol represents the mean of three different determinations, and the standard deviation was less than 6% of the mean.
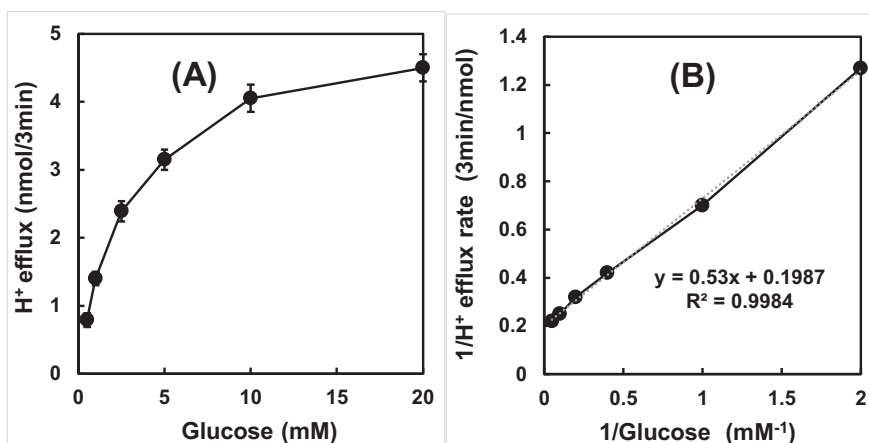
Figure 3. Kinetics of proton efflux. (A) shows proton efflux depending on glucose concentration. (B) shows Lineweaver-Burk Plots of proton efflux rate and glucose concentration. Each symbol represents the mean of three different determinations, and each bar represents the standard deviation.
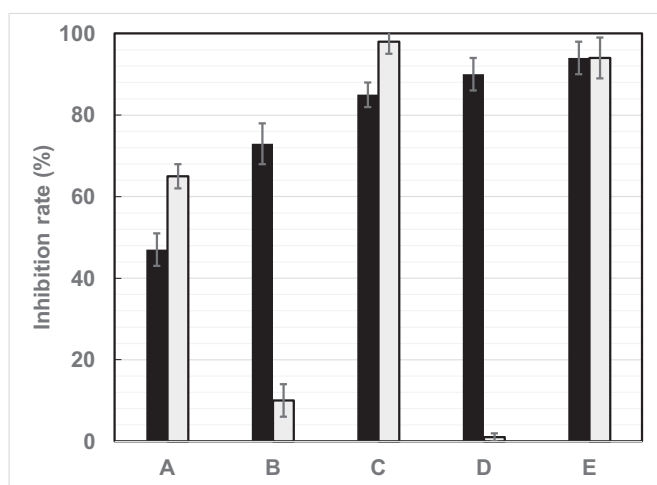

Figure 4. Effects of metabolic inhibitors. (A) 20 mM 2Deoxy-glucose, (B) 20 mM malonate, (C) 0.2 mM. Diethylstilbestrol, (D) 20 mM glucosamine, (E) 20 mM iodoacetate. Black bars and gray bars show the inhibition rate determined by methyl red test and cell proliferation measurement, respectively. Each big bar → the mean of three different determinations, and each small bar represents the standard deviation.
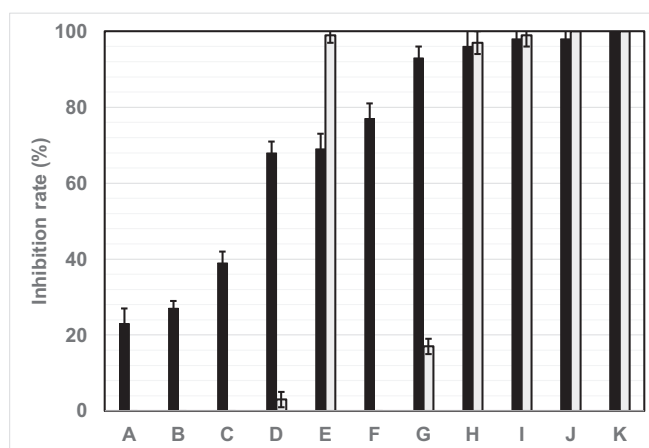
Figure 6. Effects of detergents. (A) n-Dodecyl-β-D-maltoside (0.17 mM), (B) Sodium cholate (14 mM), (C) deoxyl-BIGCHAPS (1.4 mM), (D) Triton X-100 (0.24 mM), (E) Nonidet P-40 (0.2 mM), (F) CHAPS (8 mM), (G) Sucrose mon-olaurate (0.4 mM), (H) SDS (6 mM), (I) Digitonin (0.5 mM), (J) N-laur-oylsarcosine (1 mM), (K) n-Octyl-β-D-thioglucoside (9 mM). Number in parentheses is critical micelle concentration. Black bars and gray bars show the inhibition rate determined by methyl red test and cell proliferation measure-ment, respectively. Each big bar and small bar represent the mean of three different determinations and the standard deviation, respectively.
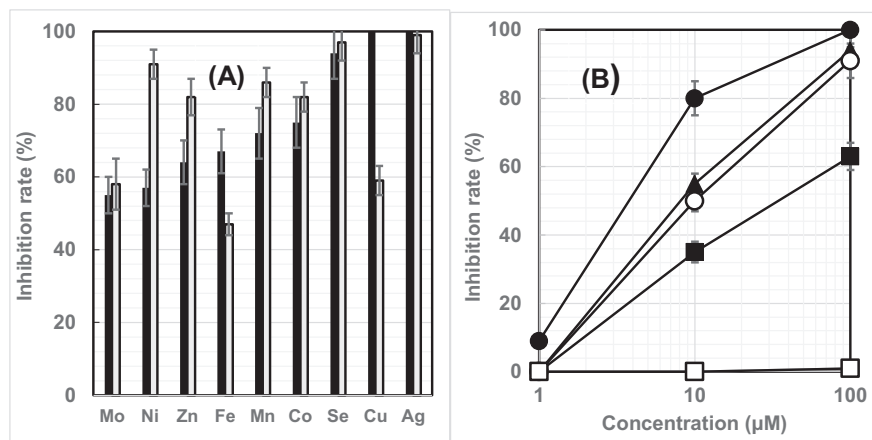
Figure 5. Effects of metal ions. The final concentration of metal ions was 10 mM in (A). The following metal compounds were tested. Mo ($Na_2MoO_4 \cdot 2H_2O$), Co ($CoCl_2 \cdot 6 H_2O$), Ni, ($NiCl_2 \cdot 6 H_2O$), Fe ($FeCl_3 \cdot 6 H_2O$), Cu ($CuSO_4 \cdot 5 H_2O$), Zn ($ZnSO_4 \cdot 7 H_2O$). Mn ($MnSO_4 \cdot 7 H_2O$), As ($NaAsO_2$), Ag (AgNO3). In (A) black bars and gray bars show the inhibition rate determined by methyl red test and cell proliferation measurement, respectively. Symbols in (B) are as follows: ●○, Ag; ▲△, Cu; ■□, As. Black symbols and white symbols show the inhibition rate determined by methyl red test and cell proliferation measurement, respectively. Each big bar and symbol represent the mean of three different determinations, and each small bar represents the standard deviation.
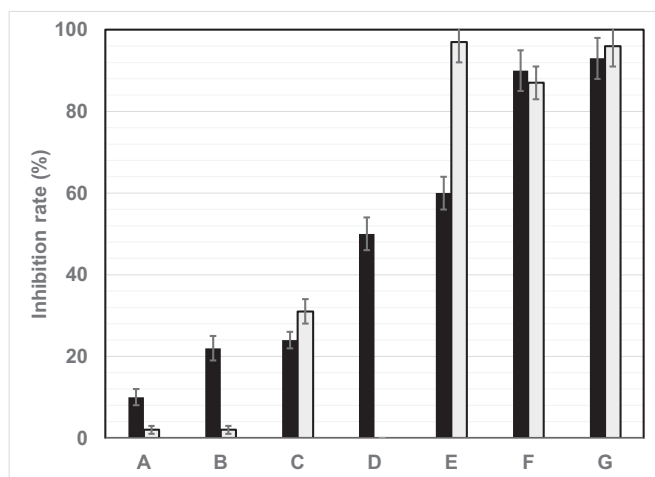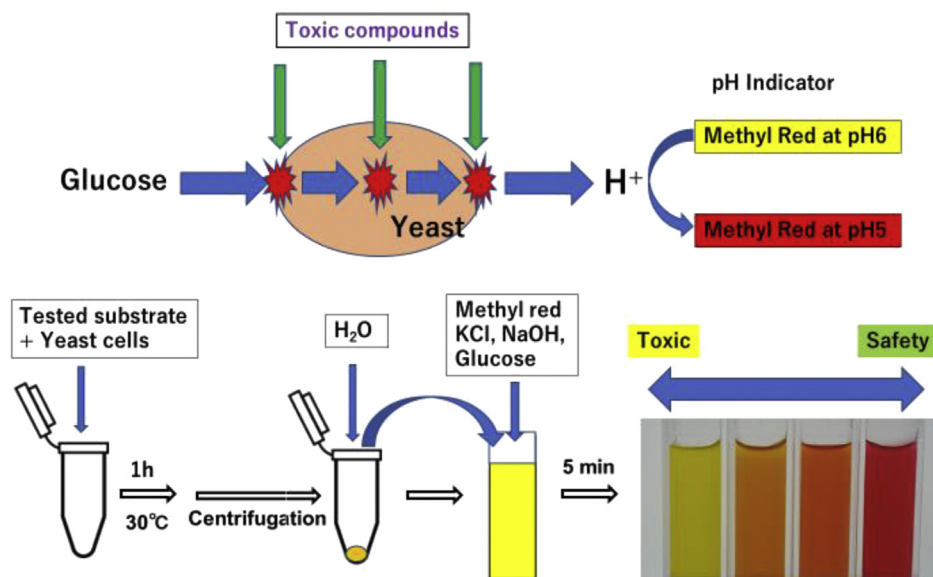
Figure 7. Effects of quinones. The concentration of quinones was 100 μM. (A)2-Hydroxy-1,4-naphthoquinone, (B)2-Methyl-1,4-naphthoquinone sodium bisulfite, (C)2-Methyl-1,4-naphthoquinone, (D)5-Hydroxy-1,4-naphthoquinone, (E)5-Hydroxy-2-methyl-1,4-naphthoquinone, (F) 5,8-Dihydroxy-1,4-naphthoquinone, (G) 1,4-Naphthoquinone. Black bars and gray bars show the inhibition rate determined by methyl red test and cell proliferation measurement, respectively. Each big bar and small bar represent the mean of three different determinations and the standard deviation, respectively.

as shown in Figure 1 (A), suggesting proton release by H^+/K^+ exchanger. After that glucose was added to yeast cell suspension, and the absorbance increased after the short lag phase. The color changed from orange to pink with increasing absorbance as shown in Figure 1 (B).

The yeast cells exposed to 2-hydroxy-1,4-naphthoquinone showed the slower increase in absorbance as shown in Figure 1 (A), and the color was orange within 5 min (not shown here). Thus, the inhibitory effect of 2-hydroxy-1,4-naphthoquinone on glucose-induced acidification was observed by methyl red test within 5 min.

Though the azo dyes such as methyl red are known to be decomposed by yeast (Jafari et al., 2014), the increase in absorbance of methyl red due to glucose-induced acidification was observed for 5 min, and the fading of methyl red was observed 30 min after methyl red test (data not shown here). Therefore, the methyl red test completed within 5 min was not affected by the decomposition of the dye by yeast cells.

3.2. Estimation of proton efflux rate

Figure 2 (A) shows the change in pH and absorbance after the addition of glucose, and Figure 2 (B) shows the correlation between absorbance and H^+ concentration calculated from pH of Figure 2 (A). In this study the proton efflux rate was calculated from the relational expression shown in Figure 2 (B).

Figure 3(A) shows the relation between glucose-induced acidification and the concentration of glucose, and Figure 3 (B) shows the plots of $1/v$ vs. $1/[S]$ in Lineweaver-Burk Plots. V_{max} and K_m were estimated to be 5.0 nmol/3min and 2.67 mM, respectively, based on Lineweaver-Burk Plots. The above results suggest that methyl red test is valid for the evaluation of plasma membrane H^+-ATPase activity and/or other enzymes supporting proton efflux.

3.3. Effects of inhibitors on glucose-induced acidification

2Deoxy-glucose is known to inhibit the intake of glucose. Malonate, glucosamine, and iodoacetate inhibit glyceraldehyde 3-dehydrogenase, hexokinase, and succinate dehydrogenase, respectively (Myers et al., 2005). Methyl red test demonstrated that these inhibitors at mM level inhibited glucose-induced acidification as shown in Figure 4. However, cell proliferation measurement showed little inhibitory effects of malonate and glucosamine, indicating the advantage of methyl red test.

Diethylstilbestrol is known to be a specific inhibitor of plasma membrane H^+-ATPase (Serrano, 1980; Eilam et al., 1984). In this study 0.2 mM diethylstilbestrol inhibited glucose-induced acidification as shown in Figure 4, but other study using high cell density (2g dry yeast/100ml) showed little inhibitory effect in the presence of 0.4mM diethylstilbestrol (Myers et al., 2005). As the inhibitory effect of diethylstilbestrol is considered to be diluted in high cell density rather than low cell density, the cell density is an important factor for the sensitive detection of cytotoxicity.

3.4. Effects of heavy metals

Metals ions are known to be involved in the various cytotoxic effects such as oxidative stress (Jomova and Valko, 2011), and damage of proteins (Rubino, 2015) and DNA (Angele-Martinez et al., 2014).

The inhibitory effects of heavy metal ions at 10 mM were observed by methyl red test and cell proliferation test as shown in Figure 5(A). Methyl red test showed the dose-dependent inhibitory effects of Ag, Cu, and As at

Figure 8. Summary of methyl red test. Intact yeast cells showed glucose-induced acidification, and color of methyl red changed from yellow to pink. Yeast cells damaged by toxic compounds showed low or no glucose-induced acidification, and the change in color was small or little detected. The procedure of methyl red test consisted of the incubation of yeast cells with test substances for 1h, the collection of yeast cells, the addition of methyl red, KCl, NaOH and glucose to yeast cell suspension, and the observation of color changes within 5 min.

the range from 1μM to 100μM, but cell proliferation measurement showed no inhibitory effects of Cu and As below 100μM as shown in Figure 5 (B), indicating the advantage of methyl red test.

3.5. Effects of detergents

Detergents are also typical pollutants and showed the inhibitory effects depending on the target site such as membrane proteins or lipids.

Figure 6 shows the inhibitory effects of detergents which are used to extract membrane proteins at critical micelle concentration. Methyl red test detected the inhibitory effects of all tested detergent at critical micelle concentration. Sodium cholate and Triton x-100 interacting with membrane lipids showed small inhibition of glucose-induced proton efflux. On the other hand, digitonin and sodium dodecyl sulfate (SDS) used as protein denaturant had strong inhibitory effects. These facts suggest that the inhibitory effects of these detergents depend on the modification and/or destruction of proteins rather than lipids. On the other hand, cell proliferation measurement had little ability to detect the inhibitory effects of n-Dodecyl-β-D-maltoside, sodium cholate, BIG-CHAPS, Triton X-100 and CHAPS, indicating the advantage of methyl red test.

3.6. Effects of quinones

1,4-Naphthoquinones and their derivatives are present as natural products and act as the prooxidants generating reactive oxygen species and as the electrophiles forming covalent bonds with tissue nucleophiles (Kumagai et al., 2011). These quinones are known to mediate redox reactions in cells and to cause the oxidative stress by enhancing the production of reactive oxygen species at below mM level.

In this study the inhibitory effects of quinones on glucose-induced acidification were observed at 100μM as shown in Figure 7. Interestingly the derivatives of 1,4-naphthoquinones containing hydroxy group and/or methyl group had smaller inhibitory effects on glucose-induced proton efflux than 1,4-naphthoquinones when determined by methyl red test.

On the other hand, cell proliferation measurement had little ability to detect the inhibitory effects of 2-hydroxy-1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone sodium bisulfite, and 5-hydroxy-1,4-naphthoquinone, indicating the advantage of methyl red test.

The inhibitory effects of the above quinones are considered to depend on the inhibition of the glycolysis enzyme glyceraldehyde-3-phosphate dehydrogenase (Miura et al., 2011; Bruno et al., 2017), the production of reactive oxygen species by NAD(P)H:quinone reductase (Yamashoji, 2016), and the production of the complex with glutathione (Zadziń ski et al., 1988).

From the above results and discussion, methyl red test was demonstrated to be superior in detection of cytotoxic substances to cell proliferation measurement. The mechanism and procedure might be summarized in Figure 8. Methyl red test is expected to be a visual and rapid colorimetric cytotoxicity test for yeast cells and to be applicable to the screening test of anti-yeast agents and various toxic compounds.

Declarations

Author contribution statement

Shiro Yamashoji: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Arafat Al Mamun: Performed the experiments; Analyzed and interpreted the data.

Latiful Bari: Analyzed and interpreted the data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Ambesi, A., Miranda, M., Petrov, V.V., Slayman, C.W., 2000. Biogenesis and function of the yeast plasma-membrane H(+)-ATPase. J. Exp. Biol. 203, 155–160.

Angele-Martinez, C., Goodman, C., Brumaghim, J., 2014. Metal-mediated DNA damage and cell death: mechanisms, detection methods, and cellular consequences. Metallomics 6, 1358–1370.

Bruno, S., Uliassi, E., Zaffagnini, M., Prati, F., Bergamini, C., Amorati, R., Paredi, G., Margiotta, M., Conti, P., Costi, M.P., Kaiser, M., Cavalli, A., Fato, R., Bolognesi, M.L., 2017. Molecular basis for covalent inhibition of glyceraldehyde-3-phosphate dehydrogenase by a 2-phenoxy-1,4-naphthoquinone small molecule. Chem. Biol. Drug Des. 90, 225–235.

Eilam, Y., Lavi, H., Grossowicz, N., 1984. Effects of inhibitors of plasma-membrane ATPase on potassium and calcium fluxes, membrane potential and proton motive force in the yeast Saccharomyces cerevisiae. Microbios 41, 177–189.

Gabriel, P., Dienstbier, M., Matoulkova, D., Kosai, K., Sigler, K., 2008a. Optimised acidification power test of yeast vitality and its use in brewing practice. J. Inst. Brew. 114, 270–276.

Gabriel, P., Dienstbier, M., Sladky, P., Sigler, K., 2008b. A new method of optical detectionof yeast acidification power. Folia Microbiol. 53, 527–533.

Jafari, N., Soudi, M.R., Kermanshahi, R.K., 2014. Biodegradation perspectives of azo dyes by yeasts. Microbiol. 83, 484–497.

Jomova, K., Valko, M., 2011. Advances in metal-induced oxidative stress and human disease. Toxicology 283, 65–87.

Kumagai, Y., Shinkai, Y., Miura, T., Cho, A.K., 2011. The chemical biology of naphthoquinones and its environmental implication. Annu. Rev. Pharmacol. Toxicol. 52, 221–247.

Lecchi, S., Allen, K.E., Pardo, J.P., Mason, A.B., Slayman, C.W., 2005. Conformational changes of yeast plasma membrane H+-ATPase during activation by Glucose: role of threonine-912 in the carboxy-terminal tail. Biochemistry 44, 16624–16632.

Miura, T., Kakehashi, H., Shinkai, Y., Egara, Y., Hirose, R., Cho, A.K., Kumagai, Y., 2011. GSH-mediated S-transarylation of a quinone glyceraldehyde-3-phosphate dehydrogenase conjugate. Chem. Res. Toxicol. 24, 1836–1844.

Myers, A., Bourn, J., Poole, B., 2005. Glucose-induced acidification in yeast cultures. J. Biol. Eng. 40, 38–42.

Na, S., Perlin, D.S., Seto-Young, D., Wang, G., Haber, J.E., 1993. Characterization of yeast plasma membrane H+-ATPase mutant pmal-A135V and its revertants. J. Biol. Chem. 268, 11792–11797.

Pascual, C., Kotyk, A., 1982. A simple spectrophotometric method for determining proton release from yeast cells. Anal. Biochem. 123, 201–204.

Rubino, F.M., 2015. Toxicity of glutathione-binding metals: a review of targets and mechanisms. Toxics 3, 20–62.

Serrano, R., 1980. Effect of ATPase inhibitors on the proton pump of respiratory-deficient yeast. Eur. J. Biochem. 105, 419–424.

Sigler, K., Hoffer, M., 1991. Mechanism of acid extraction in yeast. Biochim. Biophys. Acta 1071, 375–391.

Sigler, K., Mikyska, A., Kosar, K., Gabriel, P., Matoulkova, D., 2006. Factors Affecting the outcome of the acidification power test of yeast quality: critical Reappraisal. Folia Microbiol. 51, 525–534.

Viegas, C.A., Sebastião, P.B., Nunes, A.G., Sá-Correia, I., 1995. Activation of plasma membrane H(+)-ATPase and expression of PMA1 and PMA2 genes in Saccharomyces cerevisiae cells grown at supraoptimal temperatures. Appl. Environ. Microbiol. 61, 1904–1909.

Yamashoji, S., 2016. Different characteristics between menadione and menadione sodium bisulfite as redox mediator in yeast cell suspension. Biochem. Biophys. Rep. 6, 88–93.

Zadziński, R., Fortuniak, A., Biliński, T., Grey, M., Bartosz, G., 1988. Menadione toxicity in Saccharomyces cerevisiae cells: activation by conjugation with glutathione. Biochem. Mol. Biol. Int. 44, 747–759.